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(54) Title: A METHOD FOR SPLITTING DI-SULPHIDE BONDS AND A COMPOUND OBTAINED THEREBY

(57) Abstract

There is described a method for splitting at least one disulphide bond -S-S-, where each of the sulphur atoms is di rectly bound covalently to its respective aliphatic carbon atom in an organic substance which contains at least one sucl disulphide bond, in which each bond -S-S- which is split is converted substantially to two reactive groups of the formula $-S-S-R_1$ and R_2-S-S-, where R_1 and R_2 are equal or different and each is an organic residue. Splitting of the bond is effect ed by reacting said organic substance with a mixture of a compound $R_3-S-S-R_4$ and a compound capable of existing in th tautomeric forms R_5-S-H and $HR_5' = S$ or corresponding resonance-stabilised anion forms, in which compounds the resi dues R_3, R_4 and R_5 i) are organic residues of which all are different, two are equal or all are equal, and ii) are defined in a that each of the aforesaid sulphur atoms in the compounds $R_3-S-S-R_4$ and R_5-S-H is bound to a carbon atom in an aromat ic ring and b) that under prevailing splitting conditions a compound R_3-S-H or R_4-S-H released by the reaction of th compound $R_3-S-S-R_4$ and the compound R_5-S-H exist substantially in their tautomeric forms $HR_3' = S$, $HR_4' = S$ and HR $= S$ respectively, or corresponding resonance-stabilized anion forms. In each of the groups $-S-S-R_1$ and R_2-S-S- produce by splitting the disulphide bond, R_1 and R_2 are each a residue of the group which consists of R_3, R_4 and R_5. The inventio also relates to an organic compound produced by the aforesaid splitting and exhibiting at least one group $-S-S-R_1$ and/o R_2-S-S- bound covalently to an aliphatic carbon atom, in which groups R_1 and R_2 have the aforegiven significance.

A METHOD FOR SPLITTING DI-SULPHIDE BONDS
AND A COMPOUND OBTAINED THEREBY

The present invention relates to a method for splitting at least
one disulphide bond, -S-S-, in which each of the sulphur atoms is bonded
covalently directly to a respective aliphatic carbon atom in an organic
substance containing at least one such disulphide bond, each bond -S-S-,
5   which is split, being substantially converted to two reactive groups of
the formulae -S-S-R_1 and R_2-S-S-, where R_1 and R_2 are the same or
different and each is an organic residue. The invention also relates to
an organic compound which exhibits at least one group -S-S-R_1 and/or
R_2-S-S- bound covalently to an aliphatic carbon atom, in which groups
10 R_1 and R_2 have the aforesaid significance.

Aliphatic thiol groups (i.e. the thiol group is bound to an ali-
phatic carbon atom) and disulphide groups derived therefrom have an
important part to play in biochemical contexts. Thiol groups react under
physiological conditions with a multiplicity of other functional struc-
15 tures, such as active thioesters for example. Thiol groups are often
found in the active sites of enzymes, in which case they play an import-
ant part in the catalytic activity of the enzyme. Disulphide groups are
often present in polypeptides (which term is also considered to include
proteins) and usually appear to act as fixing elements and elements which
20 afford conformation. The part played by natural disulphides as a reagent
in the context of dynamic biochemical reactions is relatively unknown,
but must be considered to be of great significance, for example through
the aforementioned conformational properties in connection with proteins
(c.f. for example FEBS Letters 97 [1979] pages 201-10).

25 The type of reaction involving both thiol groups and disulphide
groups at the same time is designated thiol-disulphide-exchange and can
be written as:

$$X_1\text{-SH} + X_2\text{ -S-S- } X_3 \rightleftharpoons X_2\text{ -S-S-}X_1 + X_3\text{ - SH}$$
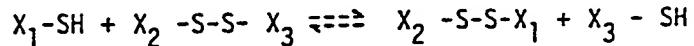
30 where X_1, X_2 and X_3 are organic residues which contain an aliphatic or
an aromatic carbon atom which is bound to one of the sulphur atoms
in -S-S-.

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Although the thiol-disulphide-exchange reaction takes place rapid-
ly under physiological conditions, the conditions of equilibrium are nor-
mally unfavourable for the preparative production of a given disulphide.
For example, when a polydisulphide is mixed with a thiol component, there
5   rapidly takes place a series of thiol-disulphide-exchange reactions
which result in a complex mixture of various disulphide-containing
entities. If the polydisulphide is a biologically active molecule, the
activity is affected.

The intriductions of so-called reactive disulphides, e.g. of the
10 alkyl pyridyl disulphide type, has greatly facilitated the preparation
of disulphides. By reactive disulphide is normally meant a disulphide
of increased electrophilicity and of such nature that departing thiols
are transformed to a stabilized tautomeric thioneform or the stabilized
anion thereof. This stabilization denies the departed thiols the possibi-
15 lity of taking part in reverse or retro-reactions, e.g. in the case of
alkyl pyridyl disulphide, aliphatic thiol-structures are not split off,
but thione structures are. In this way, there is obtained an equilibrium
which is favourable to the preparation of desired aliphatic disulphide.

Processes which are based on thiol-disulphide-exchange reactions
20 and involve biologically active molecule types are now used increasingly
in the conjugation of biomolecules with carrier polymers or biomolecules
with biomolecules, whereby in the former case the external properties of
the biomolecules, such as their solubility, separability, stability,
transportability, are changed to fulfil given requirements, while in the
25 latter case a multiple of bio-activities are combined and caused to co-
act, e.g. immunological properties with enzymatical properties or pharma-
cological properties with homing properties. Such preparations have found
use, or can be judged to find future use in connection with chromatograpy,
pharmaceuticals, diagnostic analysis methods, and in process technology.

30   Methodology in the preparation of disulphide conjugates by means of
reactive disulphides between components which do not contain thiol groups
has often involved the reaction of the components with particular thiol-
ating reagents, for example homocystein thiolactone and thiolimidates,
or with bifunctional reagents, for example the bifunctional reagent
35 N-succinimidyl-3-(2-pyridyldithio)-propionate. Thus this last-mention-
ed compound permits reactive disulphide to be introduced directly
into, for example, .

protein molecules, but, because of the ease of reduction, also the
introduction of thiol-groups.

If a component contains an aliphatic disulphide bond (i.e. a di-
sulphide bond between two aliphatic carbon atoms) and it is intended to
5   be conjugated as a disulphide with another component, which is quite
usual in respect of proteins, it has been possible, in principle, first
to reduce the aliphatic disulphide bond and then to react the resultant
thiol groups with reactive disulphide of the other component, (c.f. for
example Immunology Letters, 2 (1980) 151-155 or Haemotologia 14 (1981)
10   95-99).

This known method is encumbered with a number of disadvantages,
however. The reduction process is normally effected with aliphatic thiol-
compounds of low molecular weight. In order to achieve a partial reduc-
tion of a polydisulphide, a moderate concentration of low-molecular thiol
15   compound is used, thiol-disulphide-exchange reactions, however, being
also initiated within the polydisulphide molecule as mentioned above.
Total reduction of polydisulphide can be achieved with a high concentra-
tion of low-molecular aliphatic thiol (more often than not, together with
a denaturing agent). When such reaction mixtures are worked up, however,
20   the concentration conditions readily change, so that partial retro-reac-
tions take place. In addition, difficulties in avoiding oxidation of
the reaction-prone thiol structures with air are always liable to occur.
The possibility of avoiding separation at the thiol stage, and inhibiting
the equilibrium mixture with, for example dipyridyldisulphide is not
25   really practicable, since dipyridyldisulphide is consumed by the reducing
agent, and the asymmetric reactive disulphides initially formed initiate
reformation of a reduced polydisulphide component, both intermolecularly,
and also together with the reducing agent.

Sulphitolysis with sodium sulphite is an old, known method of cleav-
30   ing disulphides which has been applied in the field of protein chemistry
for the total splitting of multi-chain peptides into polypeptide fractions
(c.f. the references cited above). The methodology is difficult to
control, however, and requires the use of catalytic quantities of, for
example, cystein and oxygen. The advantage afforded is that the method
35   provides the thiol function with a protective group of S-sulphonate
structure.

A greater part of the disadvantages encountered with these earlier
known methods is eliminated by means of the present invention. Splitting
of a disulphide bond in accordance with the invention is characterized

in that splitting is effected by reacting the organic substance contain-
ing at least one, preferably aliphatic disulphide bond which is to be
split, with a mixture of a compound R_3-S-S-R_4 and a compound which can
exist in the tautomeric forms R_5-S-H and $HR_5' = S$ or the corresponding re-
5   soncance-stabilized anion form, in which compounds the residues R_3, R_4, R_5

        i) are organic residues, of which all are different, two are the
same or all are the same, and

        ii) are defined in

        a) that in the compounds R_3-S-S-R_4 and R_5-S-H, each of said
10   sulphur atoms is bound to a respective carbon atom in an aromatic ring, and

        b) that a compound R_3-S-H or R_4-S-H released through the reaction
of the compound R_3-S-S-R_4, and the compound R_5-S-H under prevailing splitt-
ing conditions substantially exist in their respective tautomeric forms
$HR_3' = S$, $HR_4' = S$ and $HR_5' = S$ or corresponding resonance-stabilized anion
15   forms; and that R_1 and R_2 are each a residue of the group which consists of
R_3, R_4 and R_5.

        The splitting according to the invention can be considered an
oxidation since in one stage said splitting oxidizes a disulphide-con-
taining compound by converting at least one of its disulphide bonds to
20   two new disulphide bonds. Previously, splitting methods have been re-
ductive, in that the splitting process has involved the reduction of one
disulphide bond to two thiol groups.

        In the aforegoing R_3-S-H, R_4-S-H and R_5-S-H represent the compounds
in their thiol-forms, and $HR_3' = S$, $HR_4' = S$ and $HR_5' = S$ represent said
25   compounds in their thione-form. A thiol-form and corresponding thione-
form is an example of thiol-thione-tautomerism which is fully analogous
with enol-keto-tautomerism. Both types of tautomerism are well known and
imply that a thiol group (hydroxyl group) which is bound to an sp^2-hybri-
dized carbon atom can be transformed intramolecularly to a thione group
30   (keto group). The transformation implies that a hydrogen has changed
place in the molecule, while the double bonds have changed position at
the same time, i.e. R_3, R_4 and R_5 only differ from R_3', R_4' and R_5' respec-
tively in respect of the location of the double bonds. The expression is
well known to all organic chemists. Both thione and ketone forms can be
35   transformed to their respective resonance-stabilized, deprotonized anion
forms by basic substances. In the case of the above-mentioned thione
forms, these anions are designated $^{\ominus}R_3' = S$, $^{\ominus}R_4' = S$ and $^{\ominus}R_5' = S$.

        Hereinafter, the unstable thiols R_3-S-H, R_4-S-H and R_5-S-H will
be referred to as thiones, since under the prevailing splitting conditions
40   they exist in their stabilized thione form or corresponding anion form.

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Splitting effected in accordance with the invention enables, in a single step, an aliphatic disulphide bond to be converted to two reactive disulphide structures which are inert during the splitting process, such as to enable isolation of at least one compound containing one of the

5   resultant reactive disulphide structures $-S-S-R_1$ and $-S-S-R_2$.

The splitting of an aliphatic disulphide bond in accordance with the invention in an organic substance containing one such bond can be illustrated schematically in the following manner:

$$\boxed{A_1 - S - S - A_2} \quad \boxed{A_1 - S - S - R_1 \qquad R_2 - S - S - A_2},$$
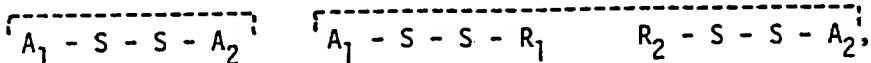
10   in which A_1 and A_2 each signify remaining parts of the organic substance, and in which the broken line signifies that A_1 and A_2 may possibly be joined together with a bond other than the $-S-S-$ bond to be split. Thus, when A_1 and A_2 are not linked by a bond other than the $-S-S-$ bond to be split, two new molecules are obtained. Thus when A_1 and A_2 are linked to

15   each other also with a bond other than the $-S-S-$ bond to be split, one new molecule is obtained. The invention also embraces an organic compound which exhibits at least one group $-S-S-R_1$ and/or R_2-S-S- bound covalently to an aliphatic carbon atom, which organic compound arises from such splitting.

20   Splitting of the aforesaid bond in accordance with the invention can be effected without needing to apply extreme pH-conditions. This enables hydrolytic side reactions to be avoided. For example, it is possible in this way to avoid hydrolysis of the disulphide bond to be split, or hydrolysis of other hydrolysis-sensitive bonds, such as amide, peptide, ketal,

25   acetal or ester bonds for example. Consequently, the invention affords a bond-splitting method which transforms an aliphatic disulphide bond into two reactive disulphide structures, in a selective and specific fashion.

The splitting reaction is pH-dependent, and can be effected in aqueous media at pH 5-12. In order to avoid the occurrence of side

30   reactions when dealing with sensitive substances, the pH-value should be within the range of 7 to 11.

The most important advantages afforded by the invention are based on the surprising discovery that the different types of disulphide bonds of a polypeptide can be split stepwise, i.e. as the excess reactive di-

35   sulphide $R_3-S-S-R_4$ and the amount of $HR_5 = S$ increases, each bond-type is split only at its own particular excess. Naturally, this will only apply when remaining conditions are kept constant. The aforesaid discovery affords important advantages preparatively, since it enables

unitary products to be obtained, i.e. products in which disulphide
bonds equivalent in type and number are split in every molecule.

Thus, splitting in accordance with the invention also provides a
method for splitting at least one disulphide bond of all the disulphide

5 bonds of a polydisulphide. Particularly important advantages are obtain-
ed when the split disulphide bond is contained in a biologically active
macromolecule, such as a protein or a polypeptide for example, which
contain, almost without exception, at least one cystine structure. In
the case of compounds such as these, the biological activity may be re-

10 tained to a greater extent than that afforded by earlier known disulphide-
bond splitting methods. The fact that it is possible to achieve such an
unusually high biological activity is probably because no intermolecular
and intramolecular thiol-disulphide-exchange reactions take place during
the process of the method according to the invention. Presumably, this

15 is because there are no aliphatic thiol groups present during the split-
ting process. If such groups are present at all, then it is only to a
very small extent. Particularly important advantages are afforded when
the polydisulphide does not contain any free thiol groups capable of
reacting with added reactive disulphide R_3-S-S-R_4. Low molecular-weight

20 thiol compounds readily initiate intramolecular thiol-disulphide-exchange
reactions. Consequently, such compounds should not be present during
the splitting process.

A large number of thiol compounds tautomerize to their thione form
spontaneously in aqueous solutions, i.e. the thione forms of such

25 compounds is more stable than their corresponding thiol form. One requi-
site in this regard is that the sulphur atom of the thiol group should be
bound to an sp^2-hybridized carbon atom in an aromatic ring. The thione
form is still further stabilized, when the aromatic ring includes a hetero-
atom (i.e. when the ring is heterocyclic), preferably nitrogen, and/or

30 is provided with at least one electron-attracting substituent selected,
for example, from the group: nitro, cyano and carboxy. The sulphur atom
is preferably located at a distance from a heteroatom in a heterocyclic
aromatic ring equal to an odd number of atoms. With respect to the split-
ting reaction, it is extremely important that the structure of the thiols

35 R_3SH, R_4SH and R_5SH is such as to permit transformation and stabilization
to respective thione form or corresponding resonance-stabilized anion
form to take place. In this respect, stabilization shall be of such a high
level that the thione and anion forms are thermodynamically denied from

thiol-disulphide-exchange reactions under the prevailing splitting
conditions. Consequently, electron-attracting substituents are always
required in the aforesaid non-heterocyclic ring systems, in order to
achieve stabilization of the thiol form to corresponding thione or anion
5   form.

Examples of R_3, R_4 and R_5 whose thiol compounds are stabilized
spontaneously under prevailing splitting conditions to corresponding
thione forms by tautomerism or resonance include: 5-nitro-2-pyridyl,
5-carboxy-2-pyridyl, 2-pyridyl, 4-pyridyl, 2-benzothiazolyl, 4-nitro-
10 3-carboxy-phenyl and the N-oxides of the above pyridyl groups. In the
reactive disulphide compounds R_3-S-S-R_4, which are preferably symmetrical,
both R_3 and R_4 are suitably selected from 5-nitro-2-pyridyl, 5-carboxy-
2-pyridyl, 2-pyridyl, 4-pyridyl, 2-benzothiazolyl, 4-nitro-3-carboxy-
phenyl and the N-oxides of the above pyridyl groups. Both reactive
15 disulphides R_4-S-S-R_3 and thions HR_5' = S, which correspond to these
groups, are readily available, and consequently are those preferred
most, for use in the splitting method of the present invention.

As will be understood, when a hydrogen atom in any of these
groups is replaced with a substituent having an aliphatic carbon atom
20 bound next to the aromatic ring, the corresponding thiol will then be
spontaneously stabilized to its thione form.

The invention also provides a method for splitting an aliphatic di-
sulphide bond in a low molecular compound which exhibits such a bond.
Examples of such compounds include cystine and carboxyl and amino deri-
25 vatives thereof. Another example is the splitting of the -S-S-bond in
thioctic acid and its carboxyl derivative. Substances which affect the
conformation of a polydisulphide can be added to the reaction mixtures,
thereby to influence the extent to which splitting takes place. For
example, when the polydisulphide is a protein, a higher degree of splitt-
30 ing can be achieved by adding urea and/or a detergent. These two sub-
stances influence the splitting process, by breaking hydrogen bonds, so
as to alter the conformation.

The disulphide bonds, which are split, are aliphatic. By this is
meant, both in the description and in the claims, that each of the
35 sulphur atoms of the bond is bound to its respective aliphatic carbon
atom to which no other atom is bound other than carbon and/
or hydrogen. Thus, those carbon atoms to which the sulphur atoms can

be bound are primary, secondary, tertiary carbon atoms, and also the
carbon atom in a methyl group.

When selecting reactive disulphide R_3-S-S-R_4 and thione, a particu-
lar advantage is afforded when the disulphide and thione chosen are such
5   that $R_3 = R_4 = R_5$. Such a choice will result in highly uniform product
mixtures ($R_1 = R_2 = R_3 = R_4 = R_5$) and utilizes readily available
reagents.

The magnitude of the practicable excess of thione and reactive di-
sulphide R_3-S-S-R_4 is primarily determined by their solubility. Disul-
10 phides and thiones which contain electrically charged groups, e.g. nega-
tively charged carboxylate groups, are more soluble in aqueous media
than those which are not electrically charged. Consequently, when a
large number of disulphide bonds are to be split it is more advantageous
to select reactive disulphides R_3-S-S-R_4 and thiones which are highly
15 soluble.

When splitting of disulphide bonds is effected in non-polar sol-
vents, non-polar disulphides R_3-S-S-R_4 and thiones are preferably
selected, since these have the greatest solubility in such solvents.

The extent to which splitting is achieved is controlled by the
20 molar concentration of thione and reactive disulphide R_3-S-S-R_4 in the
reaction mixture, and by their respective concentration in relation to
the concentration of aliphatic disulphide bonds. The extent to which
splitting is effected can be increased by means of a large excess of
thione and reactive disulphide R_3-S-S-R_4.

25 Consequently, in accordance with the invention, the molar initial
concentrations of thione $HR_5^1 = S$ and reactive disulphide R_3-S-S-R_4
relative to the initial concentration of aliphatic disulphide bonding,
is so chosen as to obtain the degree of splitting intended.

In order to run the reaction, the initial concentration of reactive
30 disulphide, R_3-S-S-R_4, should be so selected as to be in a molar excess,
e.g. up to 1000 times greater than the corresponding concentration of
the aliphatic disulphide bonds. Normally, the absolute value of the con-
centration of said reactive disulphide is selected in the range of 0.1
- 400 mM. The thione concentration is normally selected so as to be
35 from 5 to 100 times greater than the initial concentration of aliphatic di-
sulphide bonds. In this regard, the thione absolute-value should lie
within the range of 0.1 - 400 mM, although in order to achieve the
desired degree of splitting within a reasonable length of time, it is

often suitable to select a thione absolute-value from the range 10-400
mM. The upper limits of the above ranges are all determined by the
solubility of the reactive disulphide and the thione in the reaction
medium. If they are highly soluble, their respective concentrations

5   can be extended beyond the limits stated, resulting in a higher possibi-
lity of a greater degree of splitting and higher splitting rates.

It will be understood, that the precise molar proportions and
quantities are contingent on the choice of R_3, R_4 and R_5, and on the
aliphatic structure of the aliphatic disulphide bond. One of average

10 skill in this field, however, is well able to establish how the reac-
tion should be controlled, with the aid of the general guide lines
given above.

Splitting of the disulphide bonds is advantageously effected in
an aqueous solvent. Various solvents can be added, in order to improve

15 the solubility of the various reagents (reactive disulphide R_3-S-S-R_4
and thion). Naturally, the solvent added must not have a deleterious
effect on the splitting process. For example, it must not impair the
aforesaid stabilization of the thiols R_3-S-H, R_4-S-H and R_5-S-H to res-
pective thiones. Neither must it be allowed to impair the biological

20 activity, when it is desired to retain such activity.

Conveniently, the splitting process is effected at a temperature
which does not favour side reactions. With respect to biologically
active substances, for example, polypeptides which contain cystine,
it is essential that the temperature selected will not cause denatura-

25 tion of the polypeptide, if the activity is to be maintained. The
number of disulphide bonds split by the process is also contingent upon
the temperature.

Substances containing aliphatic disulphide bonds, in which a di-
sulphide bond is to be split, are usually soluble in the

30 liquid phase in which splitting takes place, although insoluble sub-
stances containing aliphatic disulphide bonds can also be split in
accordance with the invention. For example, it is possible to split
aliphatic disulphide bonds which are bound covalently to an insoluble
polymer, e.g. an insoluble porous polymer containing hydroxyl and/or

35 amino groups. Examples of such insoluble polydisulphides include
agarose, dextran cross-linked with epichlorohydrin, poly (hydroxymetha-
crylates) etc. to which covalently bound aliphatic disulphide groups
are substituted. Products obtained by means of the invention can be

used within different fields, examples of which are given below.

The introduced disulphide structures R_1-S-S- and R_2-S-S- are both
reactive to aliphatic thiol groups and can therefore be used to couple
a splitting-product with a thiol-containing compound, via a thiol-di-
5   sulphide-exchange reaction. The product thus obtained is a so-called
conjugate. The term conjugate generally implies two compounds bound
together covalently.

An important type of conjugate which can be produced from the
products obtained when splitting disulphide bonds in accordance with
10   the invention (hereinafter called splitting-products) is that which is
obtained when a compound which can be indicated analytically is con-
jugated with a ligand or receptor in which at least one disulphide bond
has been split. By receptor or ligand is meant here pairs of compounds
which have a biospecific affinity for one another. Examples of such
15   pairs include: antibody-antigen (hapten), Protein A-IgG (immunocomplex),
lectin-carbohydrate, Thyroxin-TBG, enzyme-substrate, enzyme-coenzyme
etc. As will be understood, fragments of a ligand or a receptor which
have retained their biospecific affinity shall also be considered to be
a ligand or receptor.

20   The reference to a compound which can be indicated analytically is
directed to a compound which can be detected either in the conjugate
or freed therefrom. Examples of such compounds are those which are
enzymatically active (i.e. enzymes, enzyme substrates, cofactors, co-
substrates, co-enzymes, etc.) and flourescent, phosphorescent, chemi-
25   luminescent, radioactive, metal-containing compounds etc. (c.f. for
example Schall RF et al; Clin Chim 27 (1981) pages 1157-81).

The conjugates containing analytically indicatable groups can be
used to detect various substances quantitatively and qualitatively.
A conjugate between an antibody and an analytically indicatable com-
30   pound can be used, for example, to assay corresponding native anti-
bodies or the antigen or hapten against which the antibody is directed.
In this respect, the assay can be effected in a liquid sample taken
from a mammal, for example in a sample in which the substance to be
assayed is present in a dissolved form. Particular mention can be
35   made in this respect to various heterogenous and homogenous methods
set forth in the claims of US-A-3 817 837 and US-A-4 231 999, and
described as the state of the art therein. The aforesaid conjugates

can also be used to assay cell-bound or organelle-bound ligands and
receptors to which the conjugate exhibits a biospecific affinity.
This can take place, inter alia, with the aid of different microscopy
methods.

5   Another type of conjugate is that obtained when the splitting
product is permitted to react with thiol groups on an insoluble
carrier. When the splitting-product is derived from a disulphide-
containing receptor or ligand, there can be obtained conjugates which
are particularly well suited as the solid phase for affinity chromato-
10   graphy and for the afore-mentioned heterogenous assaying methods.

A third type of conjugate is that obtained when a receptor is
split in accordance with the invention and conjugated with a thiol-
containing therapeutically active compound, for example conjugates
analogous with those described in EP-A-0 023 401, EP-A-0 017 507,
15   EP-A-0 023 779. If the receptor is an antibody which is directed
against a specific type of cell (so-called target cell) such as a can-
cer cell, the conjugate when administered to mammals will selectively
bind to its target cell. The therapeutically active compound should
therefore be more efficient.

20   Among other potential types of conjugates which can be produced
can be mentioned disulphide conjugates for immunizing purposes. Such
conjugates can be produced from a protein antigen which contains
splittable disulphide bonds. Subsequent to splitting the bonds in
accordance with the invention, the conjugate is formed when the splitt-
25   ing product is reacted with a thiol-containing soluble or insoluble
carrier.

In accordance with the invention, the splitting products obtained
can also be used directly, without pre-forming conjugates. For example,
it is known from EP-A-0 064 040 and EP-A-0 063 109 that therapeutically
30   active compounds containing asymmetric reactive disulphide structures
of the type R_1-S-S-, may exhibit a prolonged therapeutical effect, com-
pared with corresponding therapeutically active compounds which do not
have this structure. Therapeutically active disulphide compounds which
can be split in accordance with the invention without irreversably de-
35   stroying the activity of the compounds can thus be transformed, in a
novel manner according to the invention, to compounds in which the
therapeutical activity may prevail for a prolonged period of time.

In the aforegoing, mention has been made of therapeutically active compounds which can be obtained by means of the present invention. Whenever these products are administered, there is delivered a thera- peutically active dosage. The preparations employed may have the form

5   of an emulsion, a solution, tablet, etc. which may optionally contain a pharmaceutically acceptable carrier and also optionally a suitable adjuvant. The conjugates are preferably administered through subcutaneous or intravenous injections. Therapeutically active splitting products can be administered by the same route, but administration by

10   other routes is also possible for said splitting products, as well as for the conjugates.

As will be understood, in the case of the splitting-products, each property incorporated per se in the structures R_1-S-S- and R_2-S-S- can be utilized directly.

15   The greatest advantages afforded by the method according to the invention over previously known methods for introducing asymmetric reactive disulphide structures are obtained in respect of biologically active polydisulphides, e.g. cystine-containing polypeptides, such as immunoglobulins, particularly antibodies. By means of the splitting

20   method according to the invention it is possible to split one and the same disulphide bond in each molecule. If only a part of the total number of bonds present are split, e.g. if one, two or three bonds are split in an immunoglobulin, such as an antibody, it is possible to retain the tertiary structure of the polypeptide to a greater extent than

25   that afforded by previously known methods. This means that in cases such as these, the splitting-products are particularly homogenous with respect to the positioning in the molecule of the introduced structures. This also results in the formation of particularly homogenous conjugates, so as to enable the production, for example, of good

30   immunoadsorbents, in which the antigen-binding parts of all antibodies are pointing in the same direction. This also applies to the other conjugates. The homogeneity can be further increased when antibodies which derive from the same cell-line are split and conjugated, i.e. when so-called monoclonal antibodies are used.

35   The aforegoing description of the various usages to which the invention can be put has only been given by way of example and is not meant to limit the scope of the invention in any way. This is also true of the Examples given hereinafter.

EXAMPLE I

Splitting of disulphide bond in IgG

Immunoglobulin of type IgG was produced from sheep, by gel-filtration of immunosorbent pure sheep-antirabbit-IgG anti-serum on allyl
5 dextran cross-linked with N,N'-methylene-bis-acrylamide (Sephacryl®
S-300 from Pharmacia Fine Chemicals AB, Uppsala, Sweden). The IgG-antibodies (28 µM) were incubated with 5 mM 2,2'-dipyridyl disulphide and different concentrations of 2-thiopyridone (see Table 1, first column) in 40 mM sodium phosphate buffer with 0.21 M sodium chloride
10 and 10% ethanol at $23^{\circ}C$ for 24 hours in a volume of 5 ml. Subsequent to the reaction, the reaction mixtures were separated on columns containing dextran cross-linked with epichlorohydrin (Sephadex® G-25M from Pharmacia Fine Chemicals, Uppsala, Sweden) in 0.1 M sodium phosphate buffer, pH 8.5, the protein fractions being collected. The con-
15 tent of groups which could be split to 2-thiopyridone was determined in accordance with Biochem J. (1968) 173, 723-737, (c.f. Table 1, second column). The following results were obtained:

TABLE 1

(2-thiopyridone) (mM)	Equivalents of 2-thiopyridyl structures per IgG - equivalent.
12.5	0.89
25	1.23
50	1.52
100	1.86
200	2.00

EXAMPLE 2

Splitting of disulphide bonds in fragments of
30 rabbit anti-human IgE-antibody of the IgG class

Rabbit anti-human IgE-antibodies of the IgG-class were prepared by immunising rabbits with Fc-fragments of human IgE(N.D.). The anti-serum was purified by immunosorption on immobilized peptide fragments

of IgE, in order to provide anti-IgE antibodies directed against the DE_2-domains of the Fc-portion of the IgE-molecule (Advances in Immunology Vol 13 (1971) (AP)). 2.07 mg of purified antibodies in 1.0 ml of sodium acetate buffer, pH 4.5, were cleaved with 50 μg pepsin (designa-

5   tion 3,4,23 from Worthington Biochemical Corporation, Freehold, New Jersey, USA) at $37^{\circ}C$ over a period of 24 hrs (Handbook of Experimental Immunology, Wein 6-20 to 6-22).

The pepsin was then destroyed by adjusting the pH of the solution to 8.0 with a 0.1 M sodium phosphate buffer, pH 11.2 (about 750 μl).

10   24 mg of 2-thiopyridone (Ega-Chemie, Steinheim, Federal Republic of Germany) and 2.4 mg of 2,2'-pyridyldisulphide (Fluka AG, Buchs, Switzerland) were added to the solution, which was then rotated "end over end" at a temperature of $23^{\circ}C$ for 18 hours. Subsequent to centrifugation, the reaction mixture was separated on a column packed with

15   approximately 200 ml Sephacryl $^{\circledR}$ S-300 in 0.3 M saline. The main protein peak having a molecular weight corresponding to 56000 g/mole, which corresponds to the molecular weight of the Fab'-fragment of IgG (Handbook of Experimental Immunology, Wein 6-20 to 6-22) was collected and concentrated under an N_2-gas environment in a 25 ml concentrating

20   cell (model 12 from Amicon Corp, Lexington, Mass, USA) on a PM-10-filter. 0.5 ml concentrate was obtained with $A_{280}= 1.09$ (1 cm cell). The content of groups which could be split to 2-thiopyridone was determined subsequent to reduction with dithiothreitol (Sigma Chemical Company, St. Louis, Mo., USA), to $17 \cdot 10^{-6}M$, through absorbance assay-

25   ing at 343 nm. Calculations showed that the degree of substitution (equivalents of groups which could be split to form 2-thiopyridone for each equivalent Fragment) was 1.3 (Biochem. J. (1978) 173, 723-737). The absorbence of the Fragment at 280 nm was taken to be the same as that of IgG $A_{280}^{1\%} = 13.8$ (1 cm cell).

30   EXAMPLE 3

Splitting of insoluble aliphatic disulphide

Agarose gel (Sepharose $^{\circledR}$ 6B from Pharmacia Fine Chemicals, Uppsala, Sweden) was treated with 1-chloro-2,3-epoxypropane (0.45 ml reagent/3 g gel) with sodium thio-sulphate and reduced with dithiothreitol, as des-

35   cribed by Brocklehurst et al. (Biochem J. 133 (1973) 573-584). The resultant thiolagarose gel (about 750 μ-equivalents of thiolgroups/g of

agarose) was then oxidized with hydrogen peroxide in water, to form a
disulphide-agarose gel. The agarose concentration was 2% (w/v), the
hydrogen peroxide concentration 0.1 M and the reaction time 60 minutes.
Subsequent to this oxidation, the agarose gel was washed in water and
5   0.05 M sodium phosphate buffer, pH 9.0, with 50% ethanol added thereto.
The disulphide gel was then allowed to react with 2-thiopyridone (1.0 M)
and 2,2'-dipyridyl disulphide (0.5 M) in 50% ethanol at pH 9.0 under
agitation for 120 minutes at a temperature of 23°C. The gel was then
washed with 50% ethanol and water. The product was analysed by assay-
10   ing 2-thiopyridone photometrically at 343 nm, after reductive splitting
(Acta Chemica Scandinavica B29 (1975) 471-74). It contained 635 µ-equi-
valents 2-thiopyridyl structures per gram of agarose.

EXAMPLE 4

Splitting of disulphide bonds in chymotrypsinogen

15   20 mg of chymotrypsinogen A (Millipore Corp., Bedford, Mass., USA)
were dissolved in 0.5 ml of 1.0 M sodium borate buffer, pH 9.0, where-
after there were added 4.5 ml of 1.86 mM 2,2'-dipyridyl disulphide-16.3
mM 2-thiopyridone. The solution was incubated at 22, 30 and 37°C for
80 hours. Subsequent to being centrifuged the reaction mixtures were
20   de-salted on columns packed with dextran cross-linked with epichloro-
hydrin (Sephadex® G-25 M from Pharmacia Fine Chemicals AB, Uppsala,
Sweden) in 0.1 M sodium phosphate buffer, pH 7.0. The result of the
reaction was established by the A_{280}-assaying and absorbence-measuring
of dithiothreitol-split 2-thiopyridone, at 343 nm (Biochem. J. (1978),
25   173, 723-737).

The following results were obtained:

TABLE 2

Equivalents thiopyridyl structures/ chymotrypsinogen equivalent	Reaction temperature °C
0.15	22
0.42	30
2.0	37

EXAMPLE 5

Splitting of disulphide bonds in fragments of IgG

Rabbit-IgG (from Miles Scandinavia, Birkerød, Denmark) was frag-
mentated with pepsin (1:100, w/w) in 0.1 M sodium acetate buffer, pH
5   4.5, at 37°C for 24 hours. The F(ab')$_2$-fragments were separated from
the reaction mixture on a column packed with allyl dextran cross-
linked with N,N'-methylene-bis-acrylamide (Sephacryl® S-300 from
Pharmacia Fine Chemicals, Uppsala, Sweden) (Handbook of Experimental
Immunology, Wein 6-20 to 6-22). The concentration of fragments was
10 determined by A$_{280}$-measurement: A$_{280}^{1\%}$ = 1.38 (1 cm cell). Solutions
of 23.3 µM F(ab')$_2$-fragments in 0.1 M sodium phosphate solution, pH 8.0,
with 2.4% ethanol and 2.0 mM 2,2'-dipyridyl disulphide (Fluka AG, Buchs,
Switzerland) and different concentrations of 2-thiopyridone, were in-
cubated over various lengths of time at 23°C. The reaction was inter-
15 rupted by de-salting on beds of dextran cross-linked with epichloro-
hydrin (Sephadex® G-25F from Pharmacia Fine Chemicals AB, Uppsala,
Sweden), the protein fraction being collected and analysed with respect
to its protein content (A$_{280}$) and to its content of reductively cleav-
able 2-thiopyridone (A$_{343}$) (Biochem. J. (1978) 173, 723-737). The sub-
20 stitution degree (= the number of equivalents of 2-thiopyridyl disul-
phide structures per equivalent fragment) was calculated.

The following results were obtained:

TABLE 3

(2-thiopyridone)	Incubation time (hr)			
(mM)	5	24	48	96
5.4	2.07	2.83	2.32	2.60
10.7	2.10	2.76	2.27	2.45
21.5	1.81	2.66	2.25	2.49

EXAMPLE 6

30 ### Splitting of disulphide bonds in oxidized glutathione

2.27 mmoles of 2-thiopyridone, 2.27 mmoles of 2,2'-dipyridyl di-
sulphide and 0.23 mmole of oxidized glutathione (from Calbiochem AG,

Lucerne, Switzerland) were dissolved in 45 ml of 33% Et OH, whereafter
2.0 M sodium hydroxide were added to a pH of 8.1. Subsequent to reac-
tion for 4 hours at 40°C, there were added 200 μl of concentrated
acetic acid, whereafter the solution was evaporated in a rotary eva-

5 porator, to obtain 4-5 ml of partially crystallized solution. The
supernatant was centrifuged at 3000 xg for 10 minutes. Remaining crys-
tals were leached with 4 ml of distilled water, whereafter the solution
was centrifuged in the manner aforedescribed. The combined, centrifugal
solutions (7.3 ml) were extracted twice with 3 ml portions of benzene,

10 whereafter the water phase was evaporated to dryness in a rotary eva-
porator. The crystals were dissolved in a mixture of 2 ml of ethanol,
1 ml of water, and 100 μl of concentrated acetic acid. 600 μl of the
solution were placed on a thin-layer plate (Merck DC-Alufolien Kiesel-
gel 40, from Merck AG, Darmstadt, Federal Republic of Germany), which

15 was developed with 10% benzene - 10% acetic acid - 60% ethanol - 20%
water. A zone on the thin-layer plate having an Rf of 0.38 was scraped
out, whereafter the powder was extracted with 4 ml of water. The solu-
tion was centrifuged at 3000 x g for 10 minutes and evaporated. The
crystals remaining after said evaporating process were dissolved in a

20 mixture of 1000 μl of ethanol, 500 μl of water, and 50 μl of acetic
acid. Undissolved material was decantered off, whereafter the solution
was evaporated to dryness in a rotary evaporator.

The product was analysed with respect to its content of groups
which could be split reductively to 2-thiopyridone (Biochem. J. (1978)

25 173, 723-737) and with respect to amino acids in accordance with the
Moore-Steins method.

It was found that the product contained one mole of reductively
splittable 2-thiopyridone for each mole of cysteine. The same applied
to the remaining amino acids of the product, i.e. glutamic acid and

30 glycine.

The product was analysed on a thin-layer plate (Merck DC-Alufolien
Kieselgel-60 from Merck AG, Darmstadt, the Federal Republic of Germany)
with 2-thiopyridone, 2,2'-dipyridyl disulphide and oxidized glutathione
as references.

35 As the mobile phase there was used 10% benzene - 10% acetic acid
- 60% etanol - 20% water. Subsequent to chromatography, the thin
layers were coloured with iodine and ninhydrin respectively.

TABLE 4

Sample	Rf	Positive colour reaction with
2-thiopyridone	0.79	iodine
2-2-dipyridyl disulphide	0.84	iodine
oxidized glutathione	0.11	ninhydrin
2-thiopyridyl glutathione	0.43	iodine, ninhydrin

All samples each gave only one stain subsequent to development of the thin layer.

EXAMPLE 7

Conjugation of fragments of antibodies with β-galactosidase: The use of the conjugate in assaying IgE - titer in serum

β-galactosidase from E. coli (Sigma 32.1.23 from Sigma Chemical Company, St. Louis, Mo., USA) was purified by covalent chromatography on a pyridyl disulphide - agarose gel (activated thiol Sepharose® 4B from Pharmacia Fine Chemicals AB, Uppsala, Sweden) (Biochem. J. 133 (1973) 573-584). The enzyme was adsorbed on the gel at pH = 8.0 and desorbed with 0.5 M mercaptoethanol with 2 mM magnesium chloride, pH 8.0. Subsequent to desorption, the enzyme was salted-off on a column packed with dextran cross-linked with epichlorohydrin (Sephadex® G-25M from Pharmacia Fine Chemicals AB, Uppsala, Sweden) in 0.2 M sodium phosphate buffer, pH 8.0, with 2 mM magnesium chloride added. A solution containing 2.36 μM enzyme having a thiol-substitution degree of 24 equivalents thiol-groups/equivalent enzyme was obtained. 1.4 ml of the enzyme solution were mixed with thiopyridyl disulphide-containing fragments of rabbit-antihuman IgE-antibodies (c.f. Example 2), for conjugation through thiol-disulphide-exchange reaction (Biochem. J. 173 (1978) 723-737). After reacting for 96 hours at +4°C, the reaction mixture was chromatographed on a column packed with 109 ml of allyl dextran cross-linked with N,N'-methylene-bisacrylamide (Sephacryl® S-300 from Pharmacia Fine Chemicals AB, Uppsala, Sweden) in 20 mM sodium phosphate buffer, pH 7.4, with 0.3 M saline and 0.02% sodium azide added. A pool having an elution volume in the range of 39.4 - 55.2 ml with A_{280} =

0.038 was collected. The pool was diluted 40 times in 20 mM sodium phos-
phate buffer, pH 7.4, with 0.3 M saline, 0.1% human serum albumin (Sigma
A 2386 from Sigma Chemical Company, St. Louis, Mo., USA), 0.1% Tween 20,
and 0.02% sodium azide added. The diluted pool was then tested with

5   components from a Phadezym® IgE PRIST Test Kit (from Pharmacia Diagnos-
tics AB, Uppsala, Sweden). The test was carried out in accordance with
the instructions given for the IgE-test, although the β-galactosidase-
anti-IgE-antibody conjugate included in the aforesaid test kit was re-
placed with the diluted pool. An EJ serum having 41430 u IgE/ml diluted

10   in the same buffer as the above conjugate was used as standard. The
Phadezym® IgE PRIST Test was run as a reference.

    Assaying of clinical serum samples having IgE-concentrations in the
region of 0.5 - 80 kU/1 gave a high correlation between the series. In
the case of the pool, non-specific adsorption of enzyme activity on the

15   cellulose matrix, included in the test as a carrier, was only half the
adsorption obtained in the reference.

                              EXAMPLE 8

                Splitting of disulphide in IgE with 5,5'-dithiobis-
                -(2-nitrobenzoic acid) and 5-thio-2-nitrobenzoic acid
                ───

20   A 76 mM solution (in 0.1 M sodium phosphate, pH 8.1) of 5,5'-di-
thiobis-(2-nitrobenzoic acid) (DTNB) was made 14 mM with respect to di-
thiothreitol (DDT) to generate in situ a solution containing DTNB and
5-thio-2-nitrobenzoic acid (TNB). The reaction mixture was 28 mM with
respect to TNB and 62 mM with respect to DTNB. A solution which was

25   0.65 mM with respect to TNB and 12 mM with respect to DTNB was prepared
in the same manner. 0.2 ml of $1.75 \cdot 10^{-4}$ M solutions of rabbit-IgG
(from Miles Scandinavia Birkerød, Denmark) in 0.1 M sodium phosphate
buffer, pH 8.0, were then added to 0.8 ml portions of the reagent mix-
tures. Reaction mixtures were de-salted after different periods of

30   time on columns packed with dextran cross-linked with epichlorohydrin
(Sephadex® G-25 Fine from Pharmacia Fine Chemicals AB, Uppsala, Sweden)
in 0.1 M sodium phosphate buffer, pH 8.0. The protein fractions were
collected, whereafter the protein content of said fractions and the
number of groups therein which could be split reductively to TNB were

35   determined. The ratio between the determined concentrations is given
as degree of substitution (number of equivalents of groups splittable

to TNB per equivalent IgG).

TABLE 5

Incubation time	Degree of substitution	
(mins)	Reaction in 0.52 mM TNB and 9.6 mM DTNB	Reaction in 22 mM TNB and 50 mM DTNB
45	0.57	3.6
85	0.77	4.1
120	1.08	4.4
180	1.34	4.7
375	1.79	5.3
19 x 60	2.32	6.0

EXAMPLE 9

Splitting of disulphides in IgG from sheep with 6,6'-dithionico-
tinic acid (C-PDS) and 6-thionicotinic acid (C-TP)

Dithiothreitol. was added to 37 mM solutions of C-PDS (from Ega-
Chemie, Steinheim, Federal Republic of Germany) in 0.1 M sodium phos-
phate of differing pH-values according to Table 6 below, to a final
concentration of 5 mM, to reduce a 5 mM fraction of the C-PDS-solution
to 10 mM of the corresponding thion, C-TP. Dithiothreitol reduces
disulphides in solution quantitatively to corresponding reduced forms
(Biochem. 3. (1964) 480). 0.1 ml of 1.44×10^{-4} M solutions of sheep-
IgG (from Miles Scandinavia, Birkerød, Denmark) in distilled water were
added to 0.4 ml portions of these reagent solutions. The solutions were
incubated for 18 hrs at $23^{\circ}C$, whereafter they were de-salted on columns
packed with dextran cross-linked with epichlorohydrin (Sephadex® G-25
Fine from Pharmacia Fine Chemicals AB, Uppsala, Sweden) in 0.1 M sodium
phosphate buffer, pH 8.1, the protein fractions being collected. The
protein content of the fractions and the amount of groups therein
capable of being split reductively to C-TP were then determined spectro-
photometrically, and the values of the degrees of substitution subse-
quently calculated. The following molar extinction coefficients were
used:

21

$E_{342} = 9400$ for C-TP

$E_{280} = 221\ 000$ for IgG

$E_{280} = 6000$ was added to E_{280} for IgG for each equivalent C-TP
introduced.

5   <u>TABLE 6</u>

Incubation - pH	Degree of substitution (equivalents of groups splittable to C-TP/IgG equivalent
6.9	1.15
7.6	2.56
7.9	2.90
8.5	3.46
9.0	3.82
9.8	4.12

10

22

C L A I M S

1.   A method for splitting at least one disulphide bond -S-S-, where each of the sulphur atoms is directly bound covalently to its respective aliphatic carbon atom in an organic substance which contains at least one such disulphide bond, in which each bond -S-S- which is

5   split is converted substantially to two reactive groups of the formulae -S-S-R_1 and R_2-S-S-, where R_1 and R_2 are equal or different and each is an organic residue, characterized by splitting said at least one bond by reacting said organic substance with a mixture of a compound R_3-S-S-R_4 and a compound capable of existing in the tautomer-

10   ic forms R_5-S-H and HR_5' = S or corresponding resonance-stabilized anion form, in which compounds the residues R_3, R_4 and R_5

   i)   are organic residues of which all are different, two are equal or all are equal, and

   ii)   are defined in

15   a)   that each of the aforesaid sulphur atoms in the compounds R_3-S-S-R_4 and R_5-S-H is bound to a carbon atom in an aromatic ring and

      b)   that under prevailing splitting conditions a compound R_3-S-H or R_4-S-H released by the reaction of the compound

20   R_3-S-S-R_4 and the compound R_5-S-H exist substantially in their tautomeric forms HR_3' = S, HR_4' = S and HR_5' = S respectively, or corresponding resonance-stabilized anion forms, and that R_1 and R_2 each constitute a residue belonging to the group consisting of R_3, R_4 and R_5.

25   2.   A method according to Claim 1, characterized in that the organic substance is a polypeptide.

3.   A method according to Claim 2, characterized in that the polypeptide is an immunoglobulin or a fragment thereof.

4.   A method according to Claim 3, characterized in that the

30   immunoglobulin is an antibody or an antibody-active fragment thereof.

5.   A method according to Claim 1 or 2, characterized in that R_1 = R_2 = R_3 = R_4 = R_5.

6.   An organic compound exhibiting at least one group $-S-S-R_1$ and/or R_2-S-S- which is bound covalently to an aliphatic carbon atom, where R_1 and R_2 are the same or different and each is an organic residue, characterized in that said compound is obtained by the splitt-

5   ing of at least one disulphide bond $-S-S-$, where each of the sulphur atoms is covalently bound directly to a respective aliphatic carbon atom in an organic substance containing at least one such disulphide bond, by reacting the organic substance with a mixture of a compound $R_3-S-S-R_4$ and a compound capable of existing in the tautomeric forms R_5-S-H and

10    $HR_5' = S$ or corresponding resonance-stabilized anion form, in which compounds the residues R_3, R_4 and R_5

   i)   are organic residues of which all are different, two are the same, or all are the same, and

   ii)   are defined in

15   a)   that each of the aforesaid sulphur atoms in the compounds $R_3-S-S-R_4$ and R_5-S-H is bound to a carbon atom in an aromatic ring and

       b)   that under prevailing splitting conditions a compound R_3-S-H or R_4-S-H released by the reaction of the compound

20          $R_3-S-S-R_4$ and the compound R_5-S-H exist substantially in their tautomeric forms $HR_3' = S$, $HR_4' = S$ and $HR_5' = S$ respectively, or corresponding resonance-stabilized anion forms, and that R_1 and R_2 each constitute a residue belonging to the group consisting of R_3, R_4 and R_5.

I. CLASSIFICATI N OF SUBJECT MATTER (If several classification symbols apply, indicate all) 8
According to International Patent Classification (IPC) or to both National Classification and IPC   3

C 07 G 7/00,   A 61 K 39/395

II. FIELDS SEAR HED

Minimum Documentation Searched 4

Classification System	Classification Symbols
IPC	C 07 G 7/00; A 61 K 39/395; C 12 N 9/96
US Cl	260:8, 112

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched 5

SE, NO, DK, FI classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT 14

Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No. 18
X	WO, A1, 82/03394 (PHARMACIA AKTIEBOLAG) 14 October 1982	6

* Special categories of cited documents: 16

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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 1	Date of Mailing of this International Search Report 1
1983-08-14	1984 -08- 17
International Searching Authority 1	Signature of Authorized Officer 10
Swedish Pat nt Office	Elisab th arlborg

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